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GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF N-ACETYL-S-(N-METHYLCARBAMOYL)CYSTEINE, A METABOLITE OF N,N-DIMETHYLFORMAMIDE AND N-METHYLFORMAMIDE, IN HUMAN URINE

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SUMMARY

A simple method has been developed for the determination of N-acetyl-S-(N-methylcarbamoyl)cysteine in human urine. Treatment of a urine sample (1 ml) with ethanol (2 ml) and potassium carbonate (1.5 g) produces ethyl N-methylcarbamate, which is extracted into ethanol and measured by packed column gas chromatography with nitrogen-sensitive detection. The limit of quantitation in human urine is 1 μg/ml and the between-sample coefficient of variation is 5–11%. Simultaneously, N,N-dimethylformamide, N-methylformamide and formamide can also be determined.

INTRODUCTION

N,N-Dimethylformamide (DMF) is an important industrial solvent, which is particularly useful in the manufacture of plastics. DMF causes adverse effects in the liver and gastrointestinal tract of workers who are occupationally exposed to it (for reviews, see refs. 1 and 2). There are good reasons to assume that the toxicity of DMF and its metabolism are linked [3,4].

Biomonitoring of persons exposed to DMF involves gas chromatographic (GC) analysis of urine samples for its N-desmethyl metabolite N-methylformamide (MF). However, it has been shown that MF is a thermal breakdown product of N-hydroxymethyl-N-methylformamide, which is the real major urinary metabolite of DMF [5]. Recently, another metabolite, N-acetyl-S-(N-methylcarbamoyl)cysteine (AMCC) has been unequivocally identified in human urine after exposure to DMF [6]. This finding seems important in view of the association between the hepatotoxic potential of monoalkylformamides and their biotransformation to N-acetyl-S-(N-alkylcarbamoyl)cysteines [7] (AMCC has also been identified as a metabolite of MF in animals [8] and man [6]).

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This paper addresses the determination of AMCC in urine samples. The method developed is based on our previous observation that AMCC can be converted via a simple procedure into stable N-methylcarbamates, which can easily be analysed by GC [6]. Hitherto there has been no suitable assay for AMCC. The spectrophotometric determination of mercapturates [9,10] is non-specific and a reported high-performance liquid chromatographic (HPLC) method for AMCC [7] suffers from insensitivity owing to the absence of a strong chromophore in the molecule. The method presented here is rapid, specific and sensitive. It is suitable for monitoring of DMF metabolism in various investigations as it allows the simultaneous determination of DMF, MF and formamide (F) together with AMCC.

EXPERIMENTAL

Chemicals

N-Acetylcysteine was obtained from Fluka (Buchs, Switzerland) and silica gel L 40/100 from Kavalier (Votice, Czechoslovakia). Ethyl N-methylcarbamate (EMC) and methyl isocyanate were prepared as described previously [6]. All other chemicals were of analytical-reagent grade, from various producers.

Synthesis of AMCC

Methyl isocyanate (60 mmol) was added at room temperature to a solution of N-acetylcysteine (40 mmol) in acetonitrile (500 ml). The mixture was kept at ca. 30°C for 4 h, the solvent was evaporated in vacuo and the residue was purified in three runs on a silica gel column [elution with benzene–acetone–acetic acid (3:2:1, v/v), then twice with benzene–acetone (1:4); instead of these mixtures, which contain carcinogenic benzene, it is possible to use chloroform–methanol–acetic acid (4:4:1) and chloroform–methanol (1:4)]. After evaporation of the solvents in vacuo, the oily product obtained was placed in a vacuum desiccator over solid sodium hydroxide; a white solid was obtained after several weeks (m.p. 156–157°C). The yield of AMCC was 15%.

Sample preparation

Aliquots (1 ml) of urine diluted with 3 parts of distilled water were mixed with $1.5 \cdot 10^{-4}$ mol/l quinoline in ethanol (2 ml) in a 10-ml centrifuge tube fitted with a glass stopper. Powdered anhydrous potassium carbonate (1.5 g) was added in a single step and the mixture was shaken for 2 min. After centrifugation at 500 *g* for 2 min, 1 ml of the ethanolic extract was transferred to another tube and one drop of 0.1% ethanolic bromophenol blue and sufficient 0.4 *M* hydrochloric acid to turn the solution yellow were added (usually 25–50 μl of the acid were required). The ethanolic extract was then mixed with anhydrous sodium sulphate (ca. 50 mg) and the tube was shaken briefly. An aliquot (1 μl) of the extract was analysed by GC.

Chromatography

The analyses were carried out using a Chrom-5 gas chromatograph (Laboratorní přístroje, Prague, Czechoslovakia) equipped with a nitrogen-selective detector and a glass column (240 cm×3 mm I.D.), packed with 5% potassium hydroxide and 10% Carbowax 20M on silanized Chromosorb W (80–100 mesh). Nitrogen was employed as the carrier gas at a flow-rate of 50 ml/min. Hydrogen and air were supplied to the detector at 16 and 400 ml/min, respectively. The temperatures of the injector, column and detector were 230, 170 and 290°C, respectively.

Calibration graph and calculation

The calibration graph was established using aqueous solutions of EMC (0–$5 \cdot 10^{-5}$ mol/l) which were treated (without further dilution) as described under *Sample preparation*, excluding the acidification. The graph was constructed by plotting the peak-height ratio (EMC to quinoline) versus EMC concentration using least-squares analysis. The AMCC concentration in a urine sample was calculated from the EMC concentration, which was obtained by interpolation from the calibration line, taking the recovery of the EMC from AMCC (see Results) and the dilution into account. For the conditions described here, the EMC concentrations determined were multiplied by a factor of 6.

If the determination of DMF, MF and F in samples is also required, the respective calibration graphs are constructed in the same way as for EMC, using calibration solutions containing DMF, MF and F in appropriate concentrations in addition to EMC [11].

Quantitation of recovery of the AMCC→EMC reaction in aqueous solutions

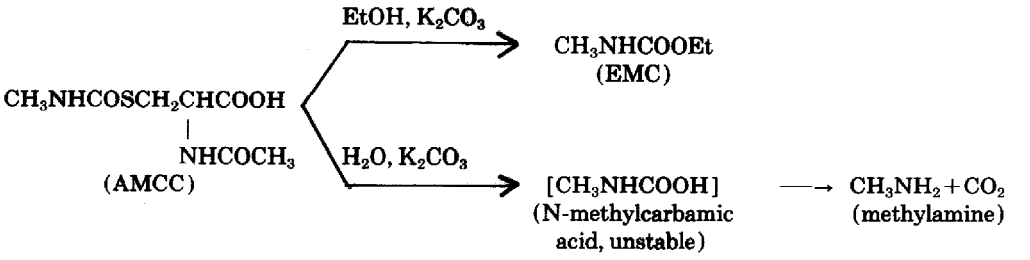
Aqueous solutions of AMCC ($1 \cdot 10^{-3}$, $1 \cdot 10^{-4}$ and $1 \cdot 10^{-5}$ mol/l) were treated (without further dilution) as described under *Sample preparation*, excluding the acidification. However, ethanol containing two internal standards, quinoline and isobutylamine, was used. For establishment of the calibration graphs, aqueous solutions of EMC ($1 \cdot 10^{-6}$–$1 \cdot 10^{-3}$ mol/l) or methylamine hydrochloride ($1 \cdot 10^{-5}$–$1 \cdot 10^{-3}$ mol/l) were treated in the same way as the AMCC solutions (methylamine is liberated quantitatively from methylamine hydrochloride owing to the alkalinity of the medium).

EMC and methylamine formed through the AMCC treatment were determined by interpolation from the respective calibration lines. The conditions relating to the EMC/quinoline measurement are described above; analysis for methylamine/isobutylamine was performed on the same column but at a temperature of only 60°C.

RESULTS

In developing the method for determination of AMCC, we focused our attention mainly on the AMCC→EMC reaction because this way of derivatization for analytical purposes is novel and its use has not been described previously. As expected on the basis of the chemical instability of N-alkylthiocarbamates in

alkaline solution, the conversion to EMC was not quantitative. Methylamine was a by-product of the reaction, which involves the intermediacy N-methylcarbamic acid. The reaction scheme is as follows:



First, the extent of the AMCC→EMC conversion (recovery, rec_{EMC}) was studied in aqueous solutions, with EMC and methylamine being determined in parallel to verify the validity of the results (Table I). When control urinary samples were analysed a small peak of an endogenous constituent coeluted with EMC. This could be eliminated by acidification of ethanolic extracts (Fig. 1). These were treated individually with 0.4 *M* hydrochloric acid until bromophenol blue, added as indicator, turned yellow. In this way the interfering peak disappeared whereas the compounds of interest were not affected. Excessive acidification, however, caused distortion of the internal standard peak.

The rec_{EMC} in control human urines (collected randomly from research institute workers) to which AMCC was added was determined, including the acidification step. As shown in Table II, the values here were generally lower than those relating the aqueous solutions and displayed considerable variability. An influence of the matrix probably accounts for these differences. Indeed, there is a significant correlation ($\alpha=0.01$) between rec_{EMC} and content of urinary components expressed in terms of creatinine concentration (Fig. 2). In order to clarify the nature of the urinary constituent which influenced rec_{EMC}, sodium chloride or urea (the major inorganic and organic urinary components) were added at concentrations of up to 5% to aqueous solutions of AMCC. The rec_{EMC} was not affected by either chemical, however. Further, rec_{EMC} in urine samples that were

TABLE I

DETERMINATION OF PRODUCTS OBTAINED FROM AQUEOUS SOLUTIONS OF AMCC ON APPLYING THE STANDARD PROCEDURE

Values given are means ± S.D. ($n=20$) for each concentration of AMCC.

AMCC (mol/l)	EMC (mol/l)	Methylamine (mol/l)	Total (mol/l)
$1\cdot10^{-3}$	$(0.75\pm0.01)\cdot10^{-3}$	$(0.24\pm0.02)\cdot10^{-3}$	$(0.99\pm0.02)\cdot10^{-3}$
$1\cdot10^{-4}$	$(0.73\pm0.04)\cdot10^{-4}$	$(0.28\pm0.05)\cdot10^{-4}$	$(1.01\pm0.02)\cdot10^{-4}$
$1\cdot10^{-5}$	$(0.69\pm0.03)\cdot10^{-5}$	Not measured	–

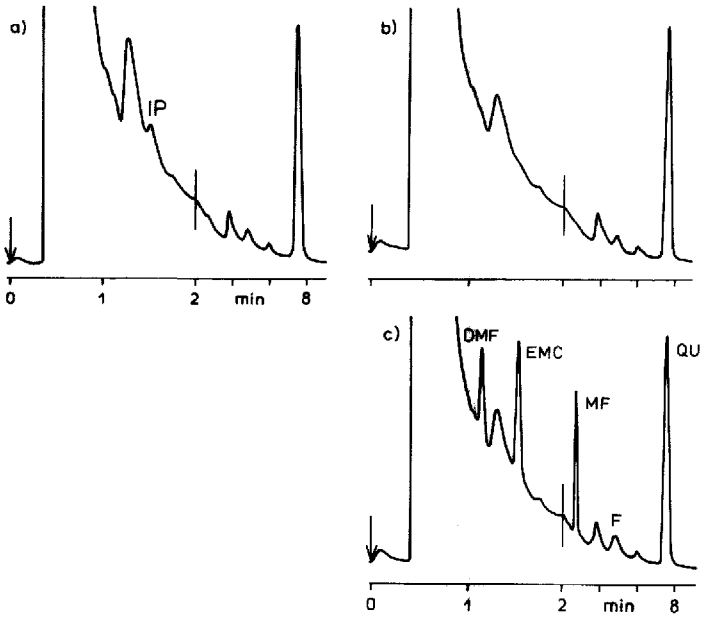


Fig. 1. Chromatograms of ethanolic extracts of control human urine (a) before and (b) after treatment with hydrochloric acid, and (c) of the same urine specimen spiked with DMF ($2 \cdot 10^{-5}$ mol/l), AMCC, MF, F ($1 \cdot 10^{-4}$ mol/l each), treated with hydrochloric acid. IP, interfering peak. Urine samples were diluted with distilled water in the ratio 1:3 before work-up. Ethanol containing quinoline as internal standard (QU, $1.5 \cdot 10^{-4}$ mol/l) was used. Attenuation: 10/16. The vertical lines through the chromatograms indicate a change of chart speed.

TABLE II

RECOVERY OF THE AMCC→EMC REACTION IN UNDILUTED URINE AND IN URINE DILUTED WITH WATER IN THE RATIO 1:3

AMCC (mol/l)	Undiluted urine			Urine diluted 1:3		
	n	rec_{EMC} (%)		n	rec_{EMC} (%)	
		Mean ± S.D.	C.V. (%)		Mean ± S.D.	C.V. (%)
$1 \cdot 10^{-3}$	60	66 ± 7	10	20	66 ± 3	5
$1 \cdot 10^{-4}$	40	62 ± 9	14	20	66 ± 4	6
$1 \cdot 10^{-5}$	40	57 ± 13	23	20	65 ± 7	11

diluted with 3 parts of distilled water after addition of AMCC was measured in order to investigate whether such dilution could improve, through the elimination of the matrix effect, the accuracy of the assay. It is obvious from Table II that the rec_{EMC} values were more consistent in this way. Dilution of the sample before work-up was therefore included in the standard procedure, the characteristics of which are given as follows.

The limit of detection of AMCC in human urine is $1 \cdot 10^{-6}$–$2 \cdot 10^{-6}$ mol/l (0.2–0.4 mg/l). The limit of determination, i.e., the concentration that can still be measured unless the result of replicate determinations ($n=6$) differs signifi-

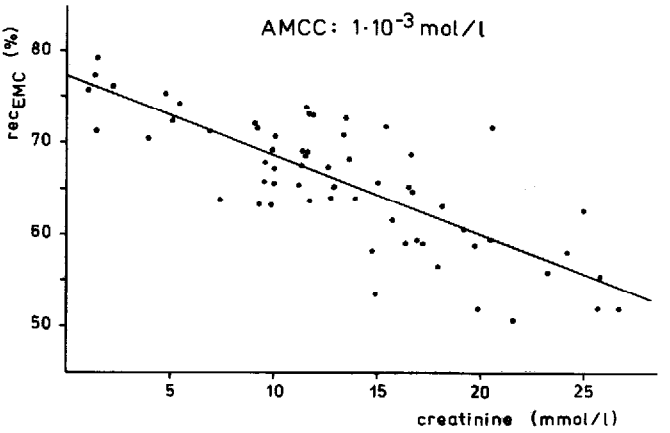


Fig. 2. Dependence of recovery of the AMCC→EMC reaction on the content of matrix components in undiluted human urine. Regression data for all AMCC concentrations measured: $c=1\cdot10^{-3}$ mol/l, $rec_{EMC}=0.773-0.0087c_{creat}$ ($n=60$, $r=-0.79$); $c=1\cdot10^{-4}$ mol/l, $rec_{EMC}=0.739-0.0100c_{creat}$ ($n=40$, $r=-0.76$); $c=1\cdot10^{-5}$ mol/l, $rec_{EMC}=0.717-0.0107c_{creat}$ ($n=40$, $r=-0.57$).

TABLE III

VALIDATION OF THE GC DETERMINATION OF AMCC IN HUMAN URINE

AMCC (mol/l)	AMCC recovery (mean ± S.D.) (%)	
	Within-day ($n=10$)	Between-day ($n=5$)
$1\cdot10^{-3}$	104 ± 3	102 ± 5
$1\cdot10^{-4}$	98 ± 5	103 ± 7
$1\cdot10^{-5}$	103 ± 7	97 ± 8

cantly (t-test, $\alpha=0.05$) from the correct value, is about $5\cdot10^{-6}$ mol/l (1 mg/l) (the corresponding values relating to aqueous solutions of AMCC are about one order of magnitude lower). The within-day and between-day reproducibilities of the method (Table III) were established using a representative blank urine to which AMCC was added. In the between-day study the samples were stored at 0°C and subjected to a single analysis on five consecutive days.

DISCUSSION

The method for the determination of AMCC in urine described here is a simple and sufficiently accurate procedure to consider its application in studies of the metabolism of DMF and in biological monitoring of occupational exposure, and it has been used successfully in recent investigations [12]. A major advantage of the method is that it offers the opportunity to measure simultaneously AMCC, DMF and the N-desmethyl derivatives of DMF, which are the thermal decomposition products of the respective N-hydroxymethyl-containing metabolites [5]. The limits of detection and determination for DMF and MF are similar to those established here for AMCC (results not shown); the characteristics of the method

for the determination of MF have been described previously [11]. The determination of F is complicated by the presence of a peak of an endogenous component, hence the respective limits for F are at least one order of magnitude higher than those for DMF and MF.

A disadvantage of the method is the dependence of rec_{EMC} on the composition of the matrix and on the AMCC concentration. First we considered determining rec_{EMC} individually for each sample on the basis of the correlations presented in Fig. 2. However, it was found that the variability of rec_{EMC} within real specimens can be improved by diluting the samples and that there was only a negligible difference between rec_{EMC} obtained for different AMCC concentrations. Hence individual determinations of rec_{EMC} could be avoided and a mean rec_{EMC} was established. In the assay used here, which adopted a dilution ratio of urine to water of 1:3, the factor which, on multiplication by the EMC concentration determined by GC, calculated the AMCC concentration in the urine sample was approximately 6.

EMC is preferable to AMCC as a standard because it is readily available in pure form.

Another small detail of the method deserves a mention as rec_{EMC} was affected by it, namely that the potassium carbonate showed be added in one step rather than in portions. Portioned addition of the salt yielded rec_{EMC} values that were significantly inferior to those obtained after the rapid addition of the whole amount, immediately followed by vigorous shaking of the sample (results not shown).

Water-soluble alcohols other than ethanol can also be used in the derivatization. The formation of 1-propyl N-methylcarbamate was helpful in experiments [12,13] where EMC coeluted with a component of the blanks that was not removable by acidification of urinary extracts. In these studies, however, AMCC was used for calibration owing to a lack of data on the recovery of the derivatization reaction.

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